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Differential Responses of Germinating *Venturia inaequalis* Conidia to Kresoxim-methyl

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Abstract: A new class of agricultural fungicides derived from strobilurins act as respiration inhibitors by binding to mitochondrial cytochrome *b*. The effects of the strobilurin, kresoxim-methyl, on conidia germination, mycelial growth and the protection of apple leaves from scab development were investigated for two isolates of *Venturia inaequalis* randomly selected from a culture collection. Inhibition of mycelial growth required relatively high doses of kresoxim-methyl (ED_{50} = 1 μg ml^{-1}) for both isolates. In comparison, germination of conidia was highly sensitive for one of the isolates (ED_{50} = 0·005 μg ml^{-1}), while the level of inhibition achieved for the second isolate was 60-fold less (ED_{50} = 0·3 μg ml^{-1}). As deduced from identical sequences of cytochrome *b* cDNAs prepared from both isolates, the different responses of germinating conidia to kresoxim-methyl were not caused by differences in the sequence of cytochrome *b* as the target site for strobilurin action. Strong synergistic effects of salicylhydroxamic acid on kresoxim-methyl inhibitory potency suggested that the reduced kresoxim-methyl sensitivity observed for germinating conidia was caused by interference of the alternative respiratory pathway with inhibitor action. However, this mechanism of target site circumvention in germinating conidia had no adverse effects on the protection of apple leaves from scab infection by kresoxim-methyl. © 1998 Society of Chemical Industry

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1 INTRODUCTION

A novel class of specific fungicides was introduced recently for the control of a broad spectrum of diseases.[1–4] The synthesis of these fungicides was guided by the structures of natural antifungal strobilurins containing a methoxyacrylate group critical for inhibitory potency.[5–8] The methoxyacrylate structure is preserved in azoxystrobin (ICIA 5504),[1] and the entire class of synthetic fungicides was named β-methoxyacrylates or MOAs.[1,9,10] Because both kresoxim-methyl (BAS 490F)[2,4] and SSF 126[3] have toxophores structurally similar but not identical to methoxyacrylate, the class is more frequently referred to as the strobilurins.[2,4,5]

Both natural and synthetic strobilurins act as respiration inhibitors by binding to the center Q_P of cytochrome *b*, an integral membrane protein forming the core of the mitochondrial bc_1 complex (complex III; ubiquinol-cytochrome *c* oxidoreductase, EC 1.10.2.2).[1,3–5,11–15] This site-specific mode of action imposes a potential risk of resistance development.[16] In *Saccharomyces cerevisiae* Meyer ex Hansen, mutational exchanges of amino acids within the strobilurin binding site of cytochrome *b* were found to decrease strobilurin sensitivities.[17–19] Similar amino acid exchanges were

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reported for the fission yeast *Schizosaccharomyces pombe* Lindner, the strobilurin-producing basidiomycetes *Strobilurus tenacellus*, *Mycena galopoda*, and for *Mycena viridimarginala*.[20] All of these organisms are naturally tolerant to relatively high strobilurin doses.[20]

The circumvention of inhibitor action by the induction of an alternative respiration pathway was described as a second mechanism of decreased strobilurin sensitivity. It had been noticed that the mycelial growth of *Pyricularia oryzae* Cav. (*Magnaporthe grisea* (Hebert) M.E. Barr)) was considerably less sensitive to the strobilurin analogue SSF 126 than respiration of isolated mitochondria.[13,21] This unexpectedly low strobilurin sensitivity was explained by the induction of an alternative oxidase rendering continued yet impaired respiration in the presence of the inhibitor.[22] A similar mechanism of target site circumvention was reported for mycelia of several other fungal pathogens such as *Botrytis cinerea* Pers. and *Monilinia fructicola* (Winter) Honey,[23] and for a laboratory mutant of *Septoria tritici* Rob.[15] In contrast to mycelial growth, conidia germination has been described as a stage highly sensitive to strobilurin fungicides.[2,4]

The strobilurin fungicide kresoxim-methyl has been introduced recently for the control of apple scab caused by *Venturia inaequalis* (Cooke) Winter.[2,24] Spore germination and host penetration was identified as the stage of apple scab development most actively controlled by the fungicide.[2] The mitochondrial cytochrome *b* gene of *V. inaequalis* was cloned and sequenced recently.[25] In this study, we describe kresoxim-methyl sensitivities of germinating conidia and mycelia for two *V. inaequalis* isolates randomly selected from our culture collection, a procedure to compare cytochrome *b* sequences derived from cDNAs, respective amino acid sequences for both isolates, and the levels of scab control achieved with kresoxim-methyl.

2 EXPERIMENTAL

2.1 Fungal isolates and materials

Two isolates of *V. inaequalis* were randomly selected from our culture collection of orchard isolates. Isolate S-56-88 was collected from an experimental orchard at the New York State Agricultural Experiment Station in 1988. Isolate KNH-6-95 was collected in 1995 from a commercial orchard in New Hampshire. Neither orchard had ever been treated with strobilurin fungicides, and the two isolates are, thus, representatives of populations never exposed to strobilurins.

Storage and culture conditions of *V. inaequalis* isolates have been described elsewhere. In brief, mycelia were stored on potato dextrose agar (PDA) covered with mineral oil.[26] Mycelial colonies were grown on PDA from agar plugs containing mycelium,[27,28] and conidia were produced on PDA covered with cellophane.[29]

Kresoxim-methyl (technical) was obtained from BASF Corporation (Research Triangle Park, NC). PDA was from Difco Laboratories (Detroit, MI). All other chemicals were from Sigma Chemical Company (St. Louis, MO), if not stated otherwise.

2.2 Tests of kresoxim-methyl sensitivities

Kresoxim-methyl and flavone were dissolved in acetone and salicylhydroxamic acid (SHAM) was dissolved in methanol. Growth media were amended with inhibitor solutions adjusted to the inhibitor concentrations to be tested. Final solvent concentration did not exceed 3 ml litre^{-1}. No effects on parameters tested were observed at these solvent concentrations.

The sensitivity of germinating conidia was tested with 30 µl of a conidial suspension (10^5 conidia ml^{-1}) in water containing various concentrations of inhibitors or solvent only. The suspensions were placed on the polystyrene surface of Petri dishes and covered with a cover glass (2·2 × 2·2 cm). Petri dishes were closed with Parafilm and incubated for 24 h at 20°C. Conidia germination was assessed for 100 conidia at each inhibitor concentration. A conidium was rated as germinated, if a normally developing germ tube had at least the length of a conidium or if an appressorium had formed at the tip of a germ tube. The sensitivity of mycelial growth was determined as described for DMI fungicides,[26–28] with the mean diameter of three colonies used in determining relative growth values for each concentration.

Protective apple scab activities of kresoxim-methyl were tested on leaves of McIntosh seedlings as described by Rich and Richards.[30] Seedlings were grown in a growth chamber at 20°C with a 14/10 h light/dark cycle at a relative humidity of 80%. A preliminary test of the pathogenicity of the two isolates was performed by inoculating leaves of five- to six-week-old seedlings with conidial suspensions (4×10^5 conidia ml^{-1}). Conidia derived from diseased seedling leaves were used in subsequent infection studies.

For sensitivity tests, both sides of susceptible seedling leaves were sprayed with various concentrations of a commercial kresoxim-methyl formulation (500 g kg^{-1} water-dispersible granule) to run-off with a spray-paint gun 24 h prior to inoculation. Concentrations were 0, 0·125, 0·25, 0·5, 1, 2, 4 and 8 µg ml^{-1}. Both sides of seedling leaves were uniformly misted with conidial suspensions (2×10^5 conidia ml^{-1}). Inoculated seedlings were incubated for 48 h at 95–100% relative humidity in a mist chamber before they were returned to the growth chamber conditions described above. Disease

development was assessed two weeks after inoculation by counting the number of sporulating lesions on both sides of the two leaves most susceptible at the time of inoculation. A total of 16 leaf surfaces was evaluated per treatment.

2.3 Preparation and sequencing of cytochrome *b* cDNAs

Mycelium of *V. inaequalis* obtained from a single colony grown for six weeks on PDA was homogenized in a Potter-Elvehjem tissue grinder with 0·5 ml RNA extraction buffer (LiCl 100 mM, Tris-HCl 100 mM pH 8·0, EDTA 10 mM, SDS 10 g litre^{-1}). The homogenate was transferred to an Eppendorf tube and extracted with an equal volume of phenol + chloroform + isoamyl alcohol (25 + 24 + 1 by mass) by vortexing for 30 s. The suspension was centrifuged, and RNA was precipitated from the supernatant by adding an equal volume of 4 M LiCl and incubating at −70°C for at least 1 h. The pellet was resuspended in 50 µl of DEPC-treated water.

Two primers were designed from flanking regions of the cytochrome *b* gene.[25] The 5′-primer P1 (5′-AGCTTATATAAAAAATCTCAC-3′) was located upstream of exon 1 and the 3′-primer P2 (5′-ATATTCATCATTTCCCATTACC-3′) downstream of exon 7 (see Fig. 3). Reverse-transcription PCR (RT-PCR) was performed using a one-step procedure[31] employing the RETROscrip kit (Ambion, Austin, TX). RT-PCR was done in 50 µl reaction buffer (Tris-HCl 100 mM pH 8·3, KCl 500 mM) containing 1 µl of total RNA prepared as described above, 1 µg of each primer, $MgCl_2$ 2·5 mM, dNTPs 0·2 mM (each), placental RNase inhibitor 5 units, MMLV reverse transcriptase 50 units, and SuperTaq DNA polymerase 1·5 units.

PCR reactions were performed in a GeneAmp PCR System 9600 thermal cycler (Perkin-Elmer, Norwalk, CT) programmed for 60 min at 42°C and 3 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, 2 min at 72°C, a final extension of 5 min at 72°C and then holding at 4°C. RT-PCR products were separated by electrophoresis on 0·7% agarose gels in 0·5 × Tris-Borate EDTA (TBE) buffer. After purification by elution of the RT-PCR products from the agarose gel using the QIAquick Gel Extraction Kit (QIAGEN, Chatsworth, CA), sequences of both strands of the RT-PCR product were determined with an ABI automated DNA sequencer with fluorescent-dye-labeled didoxy terminators at DNA Services, Cornell University. The two RT-PCR primers (P1 and P2) and two internal primers P3 (5′-CTACCTTTCGTATTAGCAGCAC-3′) and P4 (5′-CTGTCAAAGTTACCTGACACAC-3′) both located in exon 5 of the cytochrome *b* gene (see Fig. 2) were used as sequencing primers. The cDNA sequence has been deposited in the GenBank under accession number AF047029.

2.4 Data analysis

ED_{50} values were calculated by regressing percentages of inhibition against the log of inhibitor concentrations.[29] Synergistic effects of flavone on kresoxim-methyl activities were determined by testing the inhibitory activities of the two inhibitors alone and in mixture and by applying the formula $E = X + Y - XY/100$ described by Richter,[32] with E as the percentage of inhibition expected from additive effects, and with X and Y as the percentages of inhibition obtained with the two mixing partners tested alone. Synergism is apparent, if the inhibition observed for the mixture exceeds the additive effect (E) expected.

3 RESULTS

3.1 Sensitivities of two *Venturia inaequalis* isolates to kresoxim-methyl

Inhibition of mycelial growth by kresoxim-methyl required high kresoxim-methyl doses, with 50% inhibition observed at 1 µg ml^{-1} for both isolates. Inhibition of mycelial growth at 10 µg ml^{-1}, a concentration exceeding the 3·5 µg ml^{-1} water solubility of kresoxim-methyl,[4] was incomplete for both isolates (Fig. 1). Dose-responses for the inhibition of conidia germination by kresoxim-methyl were different. For isolate KNH-6-95, conidia germination was highly sensitive to the inhibitor, with 50% inhibition achieved at 0·005 µg ml^{-1}. The 60-fold higher dose of 0·3 µg ml^{-1} was required to inhibit germination of isolate S-56-88, and full inhibition of germination was not achieved at 10 µg ml^{-1} (Fig. 2).

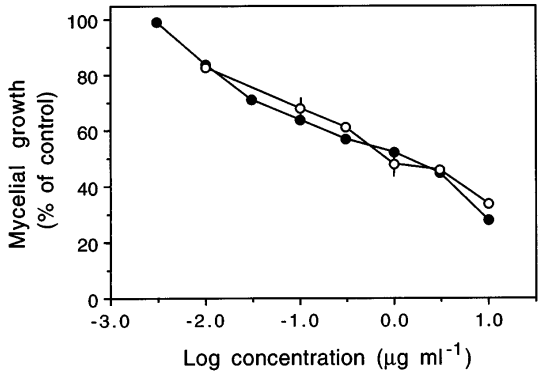


Fig. 1. Inhibitory effects of kresoxim-methyl on mycelial growth of *Venturia inaequalis* isolates (○) S-56-88 and (●) KNH-6-95. Values are the means of three separate experiments. Standard deviations exceeding the size of symbols are indicated by vertical bars.

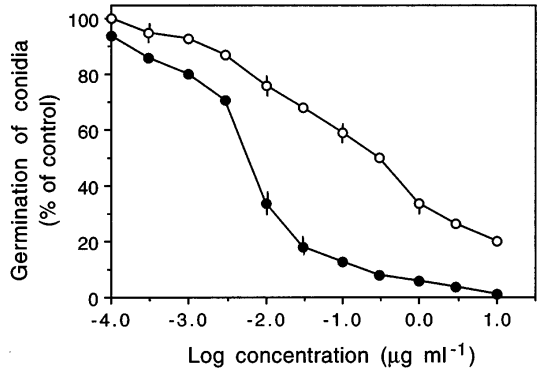


Fig. 2. Inhibitory effects of kresoxim-methyl on germination of conidia of *Venturia inaequalis* isolates (○) S-56-88 and (●) KNH-6-95. Values are the means of six separate experiments. Standard deviations exceeding the size of symbols are indicated by vertical bars.

3.2 Amino acid sequences of cytochrome *b* deduced from cDNA nucleotide sequences

The 60-fold difference of kresoxim-methyl sensitivities observed for germinating conidia of the two isolates prompted us to compare the sequences of cytochrome *b* genes. A 10·7 kb cytochrome *b* gene of *V. inaequalis* containing seven exon and six intron regions has been cloned and sequenced recently.[25] In order to rapidly compare coding regions of respective genes in isolates with different sensitivities to kresoxim-methyl, a RT-PCR method was developed for sequencing cDNAs derived from the mycelium of a single colony grown on PDA. The RT-PCR strategy is outlined in Fig. 3.

The cDNA-derived amino acid sequence determined for the fully sensitive isolate KNH-6-95 was not different from the sequence derived from the exon regions of the cytochrome *b* gene characterized before,[25] and there was no sequence departure for isolate S-56-88 (Fig. 3) indicating that the different kresoxim-methyl sensitivities displayed during conidia germination were not caused by differences in target site structures. For both isolates, amino acids reported to cause strobilurin resistance[17–20] were identical to those of fully sensitive fungi (Fig. 3).

3.3 Effects of alternative respiration on kresoxim-methyl sensitivities

A potential mechanism of strobilurin inhibitor site circumvention caused by induction of alternative respiration was investigated in experiments employing SHAM as a direct inhibitor of the alternative oxidase.[15,23] Mixtures with kresoxim-methyl were tested at a SHAM concentration of 100 μg ml^{-1} conferring 18% inhibition of conidial germination for both isolates. SHAM synergized kresoxim-methyl potency for germinating conidia of isolate S-56-88, while SHAM effects were additive for the fully sensitive isolate KNH-6-95 (Table 1). In the presence of SHAM, the responses of germinating conidia to kresoxim-methyl were similarly sensitive for both isolates (Table 1) indicating that the different kresoxim-methyl sensitivities (Fig. 2) were caused by a differential expression of alternative respiration during the stage of germination.

For several plant pathogenic fungi, the natural strobilurin tolerance observed for mycelial stages was explained by the induction of alternative respiration antagonizing strobilurin potencies.[3,22,23] In full compliance with these reports, flavone known to prevent the induction of the alternative oxidase gene in *P. oryzae* mycelium[22] synergized the inhibitory potency of kresoxim-methyl during the mycelial stage (Table 2), which was relatively low for both isolates of *V. inaequalis* (Fig. 1).

TABLE 1
Effects of SHAM[a] on Kresoxim-methyl Sensitivities of Germinating Conidia of the *Venturia inaequalis* Isolates S-56-88 and KNH-6-95

Inhibitor	*Concentration* (μg ml^{-1})	*Inhibition* (%)[b]	
		S-56-88	*KNH-6-95*
Kresoxim-methyl	0·005	21	47
	0·01	24	70
SHAM	100	18	18
Kresoxim-methyl	0·005 + 100	61 (35)[c]	58 (57)
+SHAM	0·01 + 100	71 (38)	79 (75)

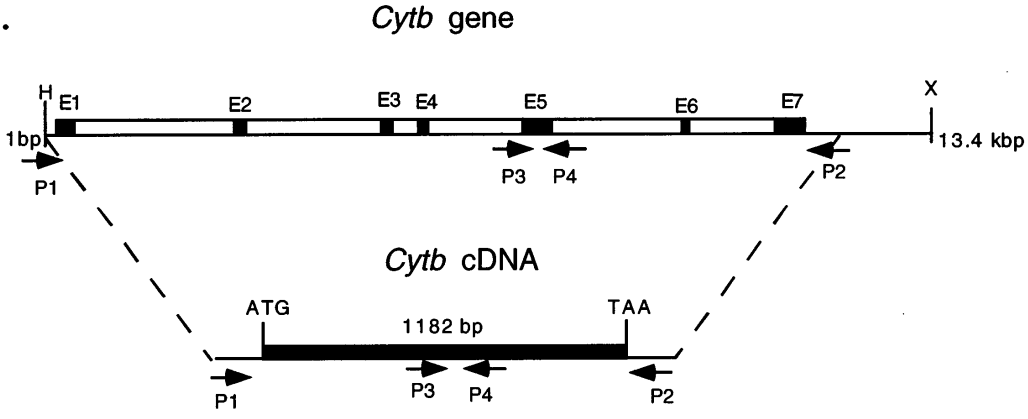
[a] Salicylhydroxamic acid.
[b] Means of four separate experiments. All standard deviations were <15% of the means.
[c] Inhibition (%) expected from additive inhibitor effects are in parenthesis.

TABLE 2
Effects of Flavone on Kresoxim-methyl Sensitivities of Mycelia of the *Venturia inaequalis* Isolates S-56-88 and KNH-6-95

Inhibitor	*Concentration* (μg ml^{-1})	*Inhibition* (%)[a]	
		S-56-88	*KNH-6-95*
Kresoxim-methyl	0·01	17	37
	1	53	46
Flavone	10	45	63
Kresoxim-methyl	0·01 + 10	82 (54)[b]	100 (69)
+Flavone	1 + 10	100 (74)	100 (80)

[a] Means of four separate experiments. All standard deviations were <20% of the means.
[b] Inhibition (%) expected from additive inhibitor effects are in parenthesis.

A.



B.

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S-56-88  :MRILKSHPLL RLANSYIIDS PQPSNISYLW NFGSLLAFCL VIQIITGVTL AMHYNPSVLE AFNSVEHIMR- 70
KNH-6-95 :MRILKSHPLL RLANSYIIDS PQPSNISYLW NFGSLLAFCL VIQIITGVTL AMHYNPSVLE AFNSVEHIMR

         DVNNGWLIRY LHANTASAFF FIVYLHMGRG LYYGSYRAPR TLVWTLGVII FILMIVTAFL GYVLPYGQMS-140
         DVNNGWLIRY LHANTASAFF FIVYLHMGRG LYYGSYRAPR TLVWTLGVII FILMIVTAFL GYVLPYGQMS
                                                                 *       *
         LWGATVITNL MSAIPWIGQD IVEFLWGGFS VNNATLNRFF ALHFVLPFVL AALALMHLIA LHDSAGSGNP-210
         LWGATVITNL MSAIPWIGQD IVEFLWGGFS VNNATLNRFF ALHFVLPFVL AALALMHLIA LHDSAGSGNP
           *   *      *
         LGVSGNFDRL PFAPYFIFKD LITIFLFILG LSIFVFFAPN ILGDSENYVV ANPMQTPPAI VPEWYLLPFY-280
         LGVSGNFDRL PFAPYFIFKD LITIFLFILG LSIFVFFAPN ILGDSENYVV ANPMQTPPAI VPEWYLLPFY
                                             * *     *              *
         AILRSIPNKL LGVIAMFAAI VILLVMPFTD LGRSRGIQFR PLSKIAYYFF IANFLILMKL GAKHVESPFI-350
         AILRSIPNKL LGVIAMFAAI VILLVMPFTD LGRSRGIQFR PLSKIAYYFF IANFLILMKL GAKHVESPFI

         EFGQISTVLY FSHFVIIVPL VSLIENTLVD LHLHNTLSLK NVF-393
         EFGQISTVLY FSHFVIIVPL VSLIENTLVD LHLHNTLSLK NVF
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Fig. 3. A, Organization of the cytochrome *b* gene of *Venturia inaequalis* and its cDNA amplified by RT-PCR. Filled boxes indicate exons (E1–E7) or the amplified cDNA, open boxes indicate intron regions, solid lines indicate flanking regions. Locations and directions of primers used in RT-PCR (P1 and P2) and sequencing (P1–P4) are indicated by arrows. H and X represent HindIII and XbaI restriction sites. B, Comparison of amino acid sequences deduced from cDNA sequences of isolates S-56-88 and KNH-6-95. Amino acids modified in strobilurin-resistant *Saccharomyces cerevisiae* mutants[17–19] or naturally resistant fungi[20] are indicated by an asterisk.

3.4 Protection of apple leaves from scab infection

Regardless of the large difference in sensitivities of germinating conidia to kresoxim-methyl, both isolates were pathogenic on apple leaves in the absence of the inhibitor (Table 3). Retests of conidia recovered from diseased leaves after two infection cycles on untreated leaves revealed that responses to kresoxim-methyl were not different from those from the conidia employed in the initial round of leaf infections, indicating that the sensitivity differences were a stable trait.

The impact of largely different kresoxim-methyl sensitivities of germinating conidia on the protection of apple leaves from scab was tested in an infection study. The protective activities determined for the two isolates are presented in Table 3. Despite the 60-fold difference of kresoxim-methyl sensitivities of conidia germination obtained under laboratory conditions (Fig. 1), protection of apple leaves from infection was not affected by this difference. Surprisingly, isolate S-56-88, with its low

TABLE 3
Effect of Kresoxim-methyl on the Protection of Apple Leaves from Infection by the *Venturia inaequalis* Isolates S-56-88 and KNH-6-95

Concentration ($\mu g\ ml^{-1}$)	*Apple scab severity*[a]	
	S-56-88	*KNH-6-95*
0	51 (16)	73 (12)
0·125	15 (7)	63 (13)
0·25	5 (2·3)	35 (12)
0·5	2 (1·6)	30 (13)
1	0·1 (0·3)	16 (4·5)
2	0	4 (1·6)
4	0	2 (2·1)
8	0	0·3 (0·7)

[a] Mean number of sporulating lesions per leaf surface. Values represent means of 16 leaf surfaces per concentration. Standard deviations are in parenthesis.

kresoxim-methyl sensitivity of germinating conidia under in-vivo conditions (Fig. 2) was more effectively controlled than the highly sensitive isolate KNH-6-95 (Table 3). The infection study was repeated twice with very similar results.

4 DISCUSSION

The results described in this study indicate a profound difference in stage-specific responses of *V. inaequalis* isolates to the strobilurin fungicide kresoxim-methyl. Considering an ED_{50} value of 0·005 μg ml^{-1} determined for germinating conidia of isolate KNH-6-95 as the full inhibitory potency of kresoxim-methyl, inhibitory potential of kresoxim-methyl was reduced by a factor of 60 in isolate S-56-88. Sequence analysis of cytochrome *b* according to a RT-PCR technique described in this study gave no indication for a target-site mutation responsible for this reduced sensitivity. Evaluation of SHAM as an inhibitor of the alternative oxidase[15,22,23,33] suggested that the inhibitory potency was reduced by interference of the alternative respiratory pathway with inhibitor action.

Circumvention of the cytochrome *b* strobilurin target site by induction of the alternative oxidase has been discussed before for mycelial stages of several pathogens.[22,23] The same mechanism was identified as the likely reason for the low mycelial strobilurin sensitivities displayed by both isolates of *V. inaequalis* described in this study. Thus far, a similar interference during the stage of spore germination has been reported only for a laboratory mutant of *Septoria tritici*.[15] For this pathogen, however, spore germination and mycelial growth were either both sensitive or both resistant to azoxystrobin.[15] A differential expression of alternative respiration during spore germination and mycelial growth as reported here for one of the *V. inaequalis* isolates investigated has not been reported before.

At present, the regulation and function of alternative respiration in fungal organisms with relevance to inhibitory potencies of strobilurin is not fully understood. In *Neurospora crassa* Shear & Dodge, this pathway involves the induction of the alternative oxidase gene and a second gene most likely regulatory in function.[33] Fine tuning of carbon flow through the mitochondrial tricarboxylic acid cycle and protection of cells from damage by active oxygen generated under conditions of a compromised cytochrome respiratory pathway have been discussed as alternative oxidase functions.[33] In isolate KNH-6-95 of *V. inaequalis*, the alternative pathway of respiration was not active during conidial germination in the presence of kresoxim-methyl, although its interference with strobilurin action became apparent during the mycelial stage. For isolate S-56-88, our results are best interpreted by suggesting that the alternative pathway was induced during both developmental stages of conidia germination and mycelial development.

The large difference in kresoxim-methyl sensitivities observed for germinating conidia was not reflected in the level of scab control achieved with the fungicide. A similar result has been reported for *Septoria tritici*.[15] Interestingly, both the *S. tritici* mutant with an alternative respiratory pathway rendering a greatly reduced strobilurin sensitivity of germinating spores[15] and the similar *V. inaequalis* isolate S-56-88 described in this study were even more effectively controlled under greenhouse conditions. Reasons for this apparent discrepancy between laboratory and greenhouse results remain to be elucidated. They might relate to the interference of host flavones with the induction of the alternative oxidase as suggested for the control of rice blast with SSF 126[22] or a reduced energy balance under conditions of alternative respiration[15,33] causing a reduction in pathogenicity.

As indicated by our results with *V. inaequalis* described in this study and a very similar observation reported for a laboratory mutant of *S. tritici*,[15] the alternative respiratory pathway responsible for the reduction of strobilurin sensitivities in the absence of the host is apparently not relevant for the level of disease control achieved with strobilurin fungicides. This pronounced discrepancy between in-vitro and in-vivo data implies that the monitoring of pathogen populations employing in-vitro sensitivity tests will be of restricted value. Resistance based on a potential mechanism of target site mutations as reported for *S. cerevisiae*, *Sch. pombe* and several basidiomycetes[17–19,33] might become more relevant in the future selection of strobilurin-resistant sub-populations of fungal plant pathogens. Thus far, mutants of this type have not been identified for any plant-pathogenic fungus. The rapid procedure of cytochrome *b* sequence analysis described in this study will aid in the search for such mutants of *V. inaequalis*.

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